

Larval and pupal sexing techniques V 2

Background:

Anopheles spp. differ from other mosquitoes in that there is often no discernable difference in the female and male larval or pupal size and general appearance. Some anophelines can be distinguished in the L4 stage based on the generally darker color and larger size of females. There is often a need to separate the sexes before they emerge e.g. in order to preserve unmated status of females, to obtain material for molecular analysis, or to determine male/female larval ratios. Here we present three methods for separating the sexes based on larval and pupal characteristics.

Larval Sexing Option 1:

Although a method has been reported for sexing *Anopheles* larvae,¹ the graphics can be difficult to interpret. Here we offer a refined method and new images developed for *Anopheles gambiae*. (The method is difficult to use with *Anopheles stephensi* whose imaginal disks are difficult to visualize.) The best results were obtained with 2-day old 4th stage larvae since the pre-antennal lobe is almost fully formed. The simple slide we describe below allows viewing the larvae without killing the larva. All observations and photographs were made on a stereoscope and it is important to use a dark-field setting.

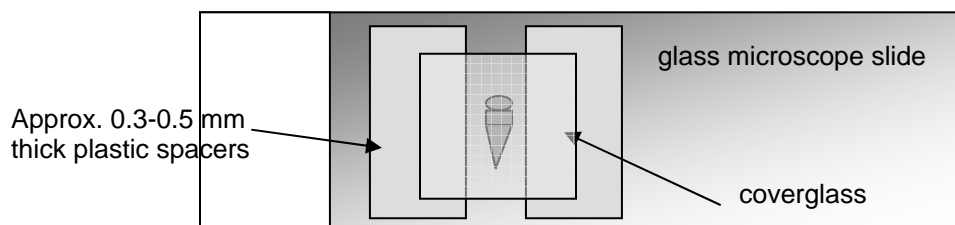
Making the viewing slide:

Materials:

- 1) Standard glass slide
- 2) 0.3 - 0.5 mm thick plastic spacer (e.g. a thin plastic laboratory ruler cut into 1 X 1.5 cm pieces). The thickness must be selected to support a coverslip over the gap so that a larva is held firmly but not crushed.
- 3) Epoxy glue

Construction:

- 1) Clean the slide with ethanol and dry.
- 2) Apply a small drop of epoxy glue to the plastic spacers.
- 3) Glue the spacers onto the slide 0.8 - 1cm apart from each other.



Place a larva, dorsum upward, between the spacers. Add sufficient water to fill the gap, and place a coverglass on top such that it bridges the spacers. When looking at the top of the larval head, the pre-antennal lobes can be seen between the imaginal eyes. In males, the lobe is large and circular and can be readily seen. The females lobe is smaller and it is easily seen only in the second and third days of the fourth instar. In our experience, males were easier to identify than females. Figure 1 is of a 2-day-old L4 male *Anopheles gambiae*. Picture 2 is of a 2-day-old L4 *A. gambiae* female. The accompanying sketches highlight the relevant portion of the photograph.

Figure 1: Male *Anopheles gambiae*

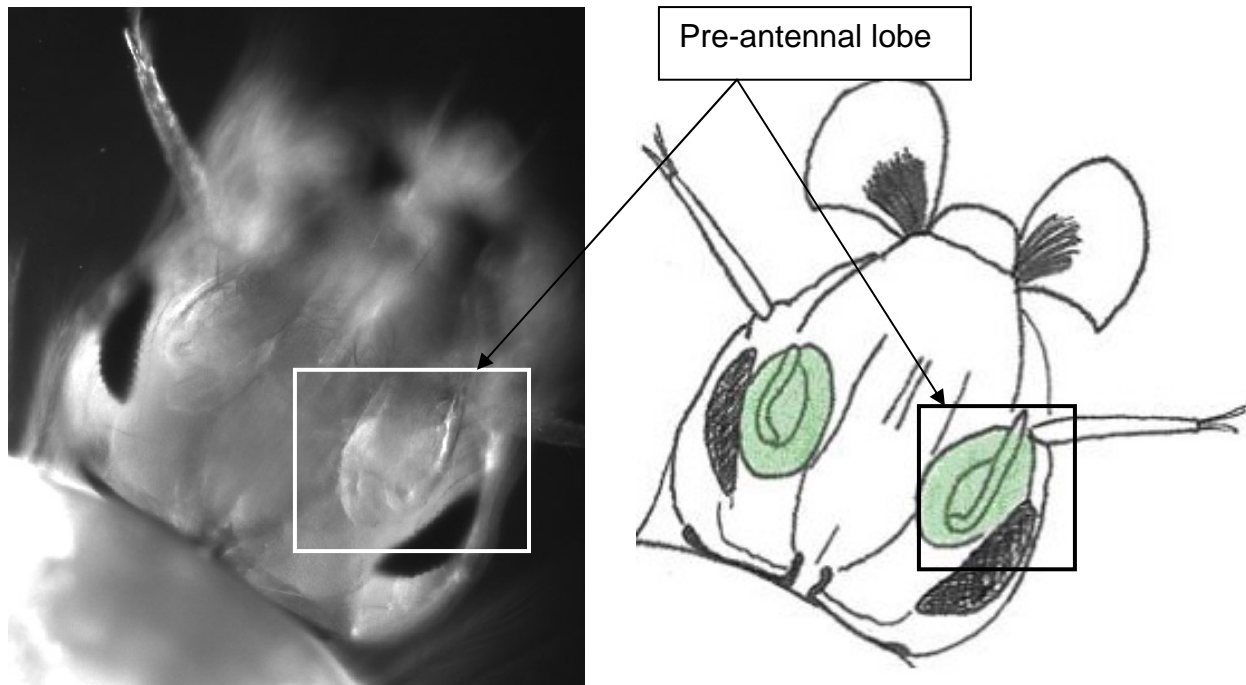
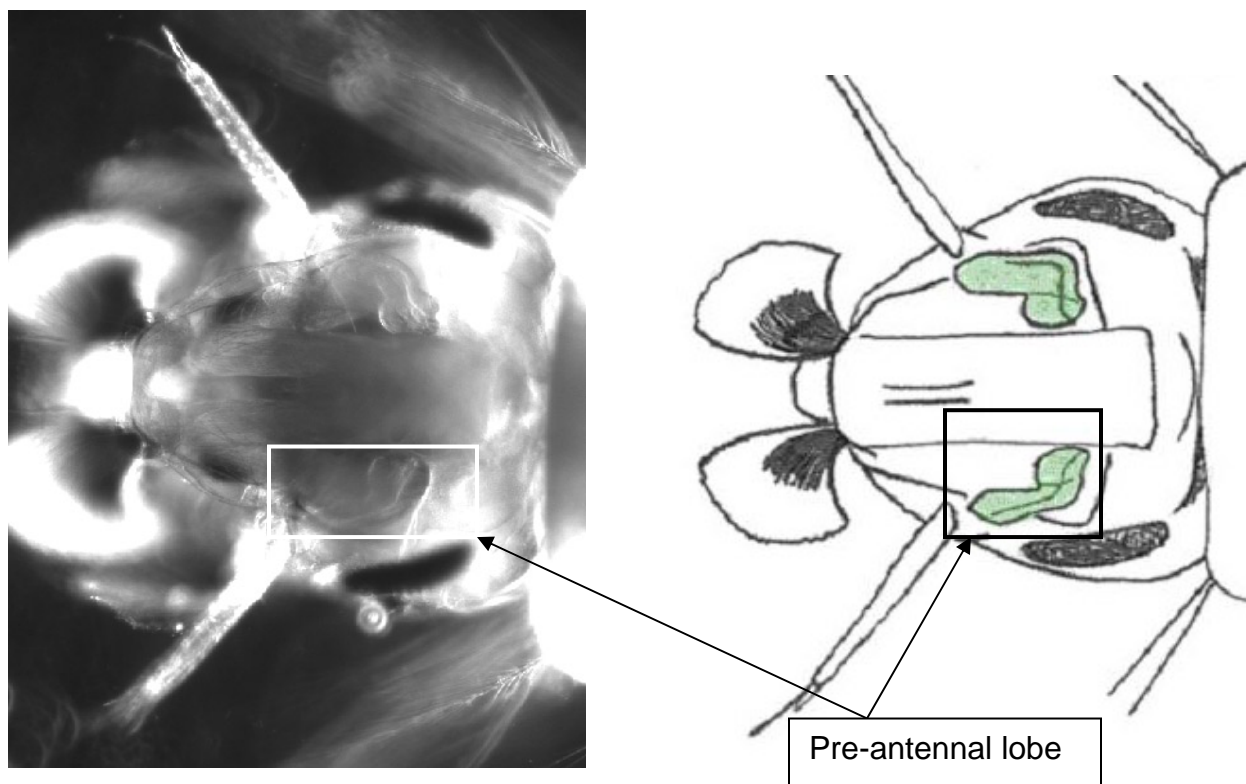


Figure 2: Female *Anopheles gambiae*

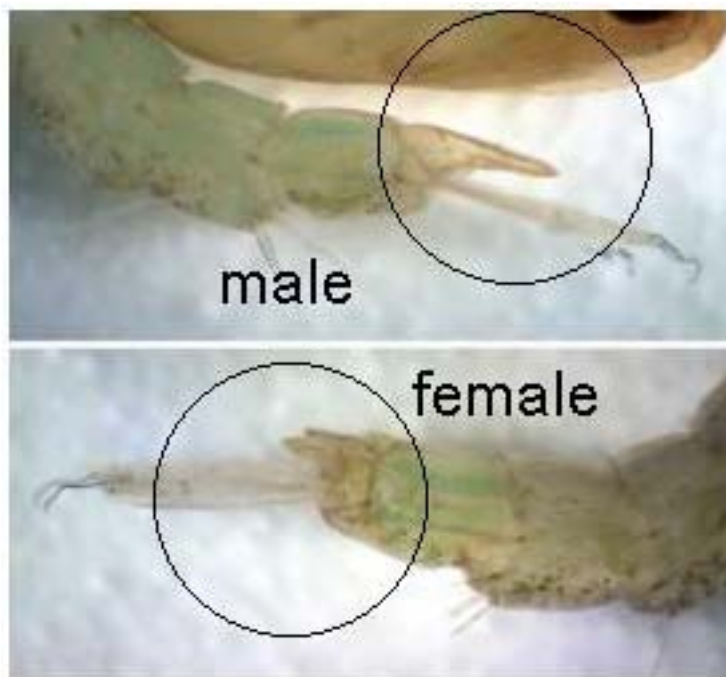


Larval Sexing Option 2:

If one is working with *Anopheles gambiae* or *arabiensis*, female L4s can often be identified by the "Red stripe" character.² When the *collarless* alleles are heterozygous ($c+ / c$), a red stripe is evident on the dorsum. This can often be seen and used to select females with high certainty. While this method does not allow one to distinguish males, a cross between a homozygous $c+ / c+$ and c / c individuals would create F1 heterozygotes in which both sexes could be distinguished with good success. The *collarless* trait is polymorphic in most colonies and wild populations and appears to have little effect on vigor. (X-chromosome markers could also be used in a genetic scheme to produce progeny whose sex could be determined as early as the L1 stage. Several such stocks are available from the MR4.) The dorsum of this L4 has white and red pigment characteristic of a ***collarless*** heterozygous female.



Pupal Sexing:



Pupae are much simpler to sex than larvae. Regardless, the pupa must be lying on its side in order to see the genitalia easily, and it may be necessary to use a small brush or forceps to gently lift the paddles.

Using a pipette, gently transfer 1 pupae to either a depression well plate or a piece of damp filter paper. If using a depression well plate, remove as much water as possible so that the pupa is lying on its side.

Under a stereoscope, observe the prominent genitalia for comparison with the figure at left.

- 1) Jones, JC. 1956. A simple method for sexing living *Anopheles* larvae (Diptera, Culicidae). **Annals of the Entomological Society of America**. 50: pp 104-106.
- 2) Benedict, MQ, LM McNitt, and FH Collins. 2003. Genetic traits of the mosquito *Anopheles gambiae*: Red Stripe, frizzled, and homochromy 1. **Journal of Heredity**. 94(3): 227-235.

* All images and drawings of mosquitoes were prepared by MR4 staff.